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(57) Abstract

The present invention relates to methods and compositions for sizing paper. In particular, the invention relates to a method of sizing paper comprising the steps of a) contacting said paper or a constituent of said paper with a protein capable of binding to said paper or said constituent of paper; and b) denaturing or heating said protein bound to said paper.

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METHODS AND COMPOSITIONS FOR SIZING PAPER

1 Technical Field

The present invention relates to methods and compositions for sizing paper. In particular, the present invention relates to the use of a protein capable of binding to paper or a constituent of paper to size paper.

2 Background

While there are a myriad of details for manufacturing paper, the paper manufacturing process conventionally comprises the following steps: (1) forming an aqueous suspension of cellulosic fibers, commonly known as pulp; (2) adding various processing and paper enhancing materials, such as strengthening and/or sizing materials; (3) sheeting and drying the fibers to form a desired cellulosic web; and (4) post-treating the web to provide various desired characteristics to the resulting paper, including surface application of sizing materials, and the like.

Sizing materials are typically in the form of aqueous solutions, dispersions, emulsions or suspensions which render the paper treated with the sizing agent, namely sized paper, resistant to penetration or wetting by an aqueous liquid, including other treatment additives, printing inks, and the like.

A sizing agent may be applied to the surface of paper as a "surface" size or may be incorporated within the paper as an "internal" size. Many chemical sizing agents are known including rosin-based and ketene dimer-based sizing compositions. There remains, however, a need for improved sizing compositions and methods of sizing.

Typically, the principal constituent of paper is cellulose. Cellulose may be in the form of wood fibre or annual crop

fibre (for example, hemp, straw, rice, flax, jute or cotton). Other constituents of paper may include other polymeric materials, including naturally occurring polymers such as starch, pectin, guar, chitin, lignin, agar, alginate as well as other polysaccharides including hemi-celluloses such as xylanose, mannose and arabinose. Xylanose is the principal component of xylan, otherwise known as hemi-cellulose which occurs in grasses, cereal, straw, grain husks and wood. Starch occurs in seeds, fruits, leaves, bulbs etc.

Enzymes which are capable of modifying an enzyme substrate typically rely on a non-covalent binding interaction with the enzyme substrate in order to function. One such class of enzymes comprise enzymes which degrade polymers, for example proteinases, keratinases, chitinases, ligninases, agarases, alginases, xylanases, mannases, amylases, cellulases and hemi-cellulases. For example, cellulases and hemi-cellulases cleave saccharide or polysaccharide molecules from cellulose and hemi-cellulose, respectively, and amylases cleave glucose from starch.

The interactions between cellulose and cellulase proteins, in particular those that bind to the cellulose fibres as a prerequisite to catalytic activity have been described and reviewed (cellulase: Béguin, Annu. Rev. Microbiol., 44, 219-248, 1990; cellulases and xylanases: Gilbert and Hazelwood, Journal of General Microbiology, 139, 187-194, 1993). This group of enzymes include cellulases and hemi-cellulases which comprise functionally distinct protein domains. In particular, the domain responsible for catalytic activity is structurally distinct from the cellulose binding domain. These domains are evolutionarily conserved sequences which are very similar in all such proteins (Gilkes *et al.*, Microbiological Reviews, 303-315, June 1991).

The binding domains of such proteins can be separated from

the active-site domains by proteolysis. The isolated binding domains have been shown to retain binding capabilities (Van Tilbeurgh, *et al.*, FEBS Letters, 204(2), 223-227, August 1986). Use of cellulose binding domains of cellulases has been proposed as a means of roughening the texture of the surface of cellulosic support, while use of cellulase active-site domains has been proposed as a means of smoothing the texture of such surfaces (International patent application WO93/05226).

A number of binding domains have also been characterised at the genetic level (Ohmiya *et al.*,Microbial Utilisation of Renewal Resources, 8, 162-181, 1993) and have been subcloned to produce new fusion proteins (Kilburn *et al.*, Published International Patent Application WO90/00609; Ong *et al.*, Enzyme Microb. Technol, 13, 59-65, January 1991; Shoseyov *et al.*, Published International Patent Application WO94/24158). Some of these fusion proteins have then been used as anchor proteins for specific applications. Such proteins have been used as an aid to protein purification through adhesion of the fusion proteins to cellulosic support materials used in protein purification strategies (Kilburn *et al.*, United States Patent 5,137,819; Greenwood *et al.*, Biotechnology and Bioengineering, 44, 1295-1305, 1994). The ability to immobilize fusion proteins onto cellulosic supports has also been suggested as a means of immobilization for enzyme bioreactors (Ong *et al.*, Bio/Technology, 7, 604-607, June 1989; Le *et al.* Enzyme Microb. Technol., 16, 496-500, June 1994), and as a means of attaching a chemical "tag" to a cellulosic material (International Patent Application WO93/21331).

It has now been found that proteins capable of binding to paper or a constituent of paper may be used to size paper.

3 Summary of the Invention

According to the present invention there is provided a

method of sizing paper comprising the steps of a) contacting said paper or a constituent of said paper with a protein capable of binding to said paper or said constituent of paper, and b) denaturing or heating said protein bound to said paper.

According to a further aspect of the present invention there is provided a method of sizing paper comprising a) contacting said paper or a constituent of said paper with a protein capable of binding to said paper or said constituent of paper, and b) heating said paper.

According to a further aspect of the present invention there is provided use of a protein capable of binding paper or a constituent of paper for the purpose of sizing paper. The invention further provides paper sized according to a method of the present invention.

4 Detailed Description of the Invention

The present invention provides a method of sizing paper. As used herein, the term "paper" refers to any material in the form of a coherent sheet or web, comprising an interlaced network of cellulose containing fibres derived from vegetable sources optionally mixed with fibres from vegetable, mineral, animal or synthetic sources in various proportions and optionally mixed with fine particles of inorganic materials such as oxides, carbonates and sulphates of metallic elements in various proportions. The term "paper" includes paperboard which refers to paper when the weight of the paper sheet or web is greater than 200g/m^2.

Vegetable sources of cellulose include wood, straws, Bagasse, Esparto, Bamboo, Kanaf, Grass, Jute, Ramie, Hemp, Cotton, Flax. The crude vegetable derived cellulose is processed to form pulp, the material from which paper is made, either mechanically, chemically or both. Cellulose containing pulps may be described as mechanical,

chemimechanical and chemithermomechanical, semi chemical, high yield chemical, full chemical (see "Pulp and Paper, Chemistry and Chemical Technology", Third Edition, Volume 1 pages 164, 165 edited by James P. Cassay ISBN 0-471-03175-5 (v.1)) according to the method of pulp preparation and purification.

Paper may also comprise other naturally occurring polymers such as proteins such as keratin, starch (including anionic, cationic or amphoteric starch), pectin, guar, chitin, lignin, agar, alginate as well as other polysaccharides including hemi-celluloses such as xylanose, mannose and arabinose.

The method of the present invention comprises contracting paper or a constituent of paper with a protein capable of binding to the paper or constituent of paper followed by denaturing or heating the protein.

The protein employed in the present invention may comprise any protein capable of binding to the paper or constituent of paper. The protein may for example comprise a protein capable of binding cellulose or any other polymeric substance present as a constituent of the paper. Preferably, the protein is capable of specific binding to cellulose or any other polymeric substance present as a constituent of paper. More preferably, the protein is capable of binding with a dissociation constant of (Kd) less than 1×10^{-3}M. As used herein, the term "protein" includes peptide, oligopeptide and polypeptide, as well as protein residues, protein-containing species, chains of amino acids and molecules containing a peptide linkage. Where the context requires (for example, when protein is bonded to another molecule), reference to a protein means a protein residue. The protein may comprise a naturally occurring protein, or fragment thereof or modified protein obtainable by chemical modification or synthesis or by expression of a genetically modified gene coding for the protein. As used

herein the term "modified protein" includes chemical analogs of proteins capable of binding to paper or a constituent thereof. Preferably, the protein comprises a naturally occurring enzyme or fragment thereof which is capable of binding to paper or a constituent of paper. Examples of proteins capable of binding paper or a constituent of paper are well known and include enzymes selected from the group comprising cellulases, hemi-cellulases, mannases, xylanases, chitinases, ligninases, agarases, alginases and amylases.

The protein may for example comprise an amylase or fragment thereof capable of binding to starch (such as anionic, cationic or amphoteric starch) when present as a constituent of paper or paper pulp. Examples of amylases include α-amylases, for example from *Aspergillus oryzae* (available as a Type X-A crude preparation from Sigma Aldrich Co Ltd), and amyloglucosidases, for example from Aspergillus niger (available from Sigma Aldrich Co Ltd).

Preferably, the protein comprises a protein capable of binding to cellulose. More preferably, the protein comprises a cellulase or fragment thereof. The cellulase may comprise a naturally occurring cellulase, or fragment thereof, or modified cellulase obtainable by chemical modification of a naturally occurring cellulase or synthesis or by expression of a genetically modified gene coding for a cellulase. The cellulase may, for example, be modified to remove or deactivate the active-site domain. A variety of cellulases are known which bind to cellulose. Examples of such cellulases are those isolable from bacterial organisms such as *Cellulomonas fimi* and fungal organisms such as *Trichoderma viride, Aspergillus niger, Fusarium oxysporum, Penicillium funiculosum, Trichoderma reesei and Humicola insolens*, available as commercial preparations from Sigma Chemical Sigma-Aldrich Company Ltd., Novo Nordisk A/S, BDH Ltd., or ICN Biomedicals Ltd. (*Fusarium oxysporum* is available for example under deposit No. DSM 2672). Alternatively, the protein may be produced by recombinant

DNA techniques as disclosed in, for example, International Patent application WO94/24158. Cellulases generally comprise a cellulase binding domain and a domain responsible for cellulase activity. The present invention may employ the cellulase as a whole or a fragment thereof capable of binding to cellulose. A cellulase binding domain may be obtained from whole cellulase by treatment with protease(s), such as papain. The cellulase may comprise an exo-cellulase or an endo-cellulase. Exo-cellulases (also known as cellobiohydrolases, CBH; exoglucanaes; 1,4-beta-D-glucan cellobiohydrolases; EC 3.2.1.91) act on the non-reducing end of a cellulose molecule. Exo-cellulases may release terminal cellobiose units (a disaccharide) or release terminal glucose units (monosaccharide). Examples of exo-cellulases include cellulase obtainable from *Humicola isolens*. Endo-cellulases (also known as Beta-1,4-Endoglucanases; Endo-1,4-D-glucanases; 1,4-Beta-D-glucan glucanohydrolases; EC 3.2.1.4) cleave internal beta-1,4-glycosidic bonds yielding a mixture of glucose, cellobiose and other soluble cello-oligosaccharides. Examples of endo cellulases include cellulase obtainable from *Trichoderma reesei*.

The protein may also comprise cellulosomes. Cellulosomes comprise a cellulase system comprising discrete, multifunctional, multienzyme complexes. They typically contain at least 14 distinct polypeptides including numerous endoglucanses (endocellulases) and xylanases and at least one beta-glucanase. These are associated with scaffolding proteins. Cellulosomes are described in detail in Bayer E.A., Morag E., Lamed R. (1994), "The cellulosome - a treasuretrove for biotechnology", Trends in Biotechnology 12:379-386.

Preferably, the protein employed in the present invention comprises cellulase obtainable from *Humicola isolens* (available as Celluzyme® from Novo Nordisk A/S, Bagsvaerd, Denmark) or cellulase obtainable from *Trichoderma reesei*

(available as Celluclast® from Novo Nordisk A/S, Bagsvaerd, Denmark). More preferably, the protein comprises cellulase obtainable from *Humicola isolens*.

The protein may be added to the paper at any suitable stage in the manufacture and processing of the paper. It may be added at the pulp stage or at any stage during the formation of the wet pulp matrix or during the pressing and rolling of the matrix to form paper. Thus, according to the present invention there is provided a method of manufacturing sized paper comprising the steps of a) preparing a paper pulp, b) adding a protein capable of binding to a constituent of said pulp, c) forming paper from said pulp, and d) heating said paper.

Alternatively, the protein may be added to the formed paper product, for example, by immersing the paper in a bath containing the protein or by any suitable spraying, spreading, brushing, coating or printing process. Thus, the invention further provides a method of manufacturing sized paper comprising the steps of a) applying to paper a protein capable of binding said paper and b) heating said paper.

By choosing the point in the manufacture of the paper at which the protein is added, control may be exercised as to whether the protein is distributed throughout the paper or is substantially restricted to the surface levels of the paper.

The protein should be incubated with the paper or paper pulp for sufficient time to allow binding of the protein to the paper or paper pulp. Typically, 15 minutes has been found adequate, although shorter incubation times may be suitable.

The protein may be added in an amount suitable to achieve the desired level of sizing. The protein may be added in an amount of 0.01-40% by weight of the dry weight of the paper pulp. Preferably the protein is added in an amount of 0.1

to 20% by weight, more preferably 1 to 10% by weight.

Following incorporation of the protein in the paper or application of the protein to the surface of the paper, sizing of the paper is achieved by denaturing or heating the protein. The protein may be denatured by the application of a chemical protein denaturant to the paper. Chemical protein denaturants include urea, guanidine, acids, alkalis, detergents (such as Tween®), water soluble organic substances (such as alphatic alcohols) and chaotropic ions (such as I^-, ClO_4^-, SCN^-, Li^+, Mg^{2+}, Ca^{2+} and Ba^{2+}).

Preferably, sizing is achieved by heating the paper. Preferably, the paper may be heated at a temperature of 50°C to 200°C, more preferably 70°C to 170°C, more preferably 80°C to 110°C, more preferably 100°C to 110°C. Typically, the paper may be heated to approximately 105°C on steam heated rollers. The paper may be subjected to one or more heat treatments at different temperatures.

The length of time of heating required depends upon the temperature at which the paper is heated, longer times being required at lower temperature. Typically, the paper may be heated for between 15 and 500 seconds, preferably between 25 and 300 seconds. The paper may also be subjected to post manufacture heat treatments to age or cure the paper.

The invention will now be described with reference to the following examples.

It will be appreciated that the following is by way of example only and modification of detail may be made within the scope of the invention.

EXPERIMENTAL

Materials and Protocols

Except as indicated below, the following materials and protocols were used in the Examples to characterise the use of proteins as biosizing agents.

Pulp: Water-leaf paper pulp was prepared by adding 10 g of water-leaf paper (70:30 Hardwood (birch) : softwood (pine)) to 100 ml distilled water. After 5 min the paper was blended to an homogenous pulp. Samples of pulp suspension (corresponding to 0.2 g dry paper) were weighed into Universal bottles.

Incubation: To each bottle 10.0 ml of one of the following incubation buffer solutions was added:

(a) Tris-HCl, 50 mM, pH 7.5
(b) Phosphate buffered saline (PBS) 500 mM, pH 7.5

Cellulases: To the incubation suspension was added a cellulase selected from the following:

1. *Humicola insolens* - Cellulase derived from *Humicola insolens* available as Celluzyme® from Novo Nordisk A/S, Bagsvaerd, Denmark (875µl corresponding to 8.7% weight per weight cellulose binding protein to dry weight pulp).

2. *Trichoderma reesei* - Cellulase derived from *Trichoderma reesei* available as Celluclast® from Novo Nordisk A/S, Bagsvaerd, Denmark (70µl corresponding to 0.28µmols protein corresponding to 4.4% weight per weight cellulose binding protein to dry weight pulp).

3. *Trichoderma viride* - Cellulose derived from *Trichoderma vivide* available from BDH Ltd UK.

Control experiments in the absence of cellulase were also performed.

Incubation: The mixtures were incubated for 15 min at room temperature with gentle agitation.

Test Sheet Preparation: To produce the paper test sheets, the volume was increased to 100 ml with distilled water and paper sheets (6 cm^2) produced using a laboratory-designed paper making apparatus operated in the following manner: a suspension of paper pulp (0.2% wv^{-1}) was poured into a plastic filter holder which houses a fine nylon filter mesh. By applying a vacuum for a few seconds the pulp was formed into a paper sheet supported by the mesh. The filter mesh was removed from the apparatus and the paper sheet sandwiched between a second nylon mesh and blotted between blotting paper. The paper sheet was carefully removed from the paper-making mesh, flattened by rolling and then dried.

Test Sheet Drying/Heating: Paper sheets were dried in one of the following ways

(a) air drying;

(b) drum drier - typically operating at a constant temperature of 80°C to 108°C with a contact time of 40 to 250 s;

(c) hot plate press - typically producing a maximum surface temperature of 160°C with a 30 s contact time. A flat aluminum plate was used to press the paper test sheets (sandwiched between blotting paper) against the hot plate.

Test protocols: The dried sheets were assessed for sizing by one or more of the following tests:-

(a) Ink drop test (IDT) - in which a 15 μl drop of Parker Super Quink Ink (Permanent Blue-Black) was dropped

onto the test piece of paper and the time taken for complete absorption measured and recorded. Results were recorded in seconds or on an empirical scale of 0 to ++++ in which:-

0 means less than 100 s for complete absorption.
+ means 100-500 s for complete absorption.
++ means 500-1000 s for complete absorption.
+++ means 1000-2000 s for complete absorption.
++++ means greater than 2000 s for complete absorption.

(b) Hercules Size Test (HST) - defined in "Tappi Test Methods" published by TAPPI, Technology Park, Atlanta, PO Box 105113, GA 30348, USA, ISBN 0-89852-200-5 (vol 1 and 2), (1987). The HST is defined as size test for paper by ink resistance T530pm-83. The data are recorded in seconds; the higher the value, the better the sizing. Preferably an HST value greater than 20 seconds, more preferably greater than 120 seconds, more preferably greater than 200 seconds, is obtained.

(c) Cobb Size Test - defined in "Tappi Test Methods" (Ibid) by T441om-84. Data are recorded in grams/m^2. "Fully saturated" means that the paper showed no sizing at all. The lower the Cobb value, the better the sizing. Preferably a Cobb value less than 30g/m^2, more preferably less than 21g/m^2, is obtained.

Example 1

An investigation into the effect of temperature on the sizing imparted by a *Trichoderma reesei* cellulase preparation (Celluclast®, Novo Nordisk A/S, Bagsvaerd, Denmark;) was carried out. For these studies a drum drier was used in conjunction with a variable temperature hot plate. The drum drier gave a constant temperature of 80°C with a contact time of 250 s and the hot plate gave a maximum surface temperature of 160°C with a 30 s contact time. A flat aluminium plate was used to press the paper sheets (sandwiched between blotting paper) against the hot

plate.

Trichoderma reesei cellulase preparation (70 µl Celluclast®, corresponding to 4.4 % ww^{-1} cellulose binding protein based on dry weight of cellulose fibre) was used and the mixtures incubated for 15 min at room temperature with gentle agitation. Control experiments without cellulase were also performed.

Paper test sheets were prepared as described above. Test sheets were initially pressed between blotting paper then dried in one of the three following ways.

(a) air drying;
(b) drum-drier: 80°C/250 s; or
(c) hot plate press: 160°C/30 s followed by drum drier 80°C/250 s.

The dried sheets were then assessed for sizing by the Ink Drop Test (IDT).

The following sizing results were obtained from test papers prepared under different drying regimes.

Table 1: Effect of paper drying regime on the cellulase-based sizing system

Additions to pulp	Drying regime		
	Air dried	80°C/250 s	160°C/30 s + 80°C/250 s
Celluclast®+ Tris-HCl	0	+++	++++
Celluclast® + PBS	-	-	++
Tris-HCl	-	0	0
PBS	-	0	0
H_2O	-	0	0

- = Not tested.

No sizing was observed when papers were made without enzyme, ruling out the possibility that the buffer salts were interacting with the cellulose in such a way as to affect sizing. Sizing was observed with Tris-*T. reesei* cellulase

(Celluclast®) incubations and a greater degree of sizing was observed when papers were dried initially at the higher temperature followed by drum drying at the lower temperature. Cellulase/PBS paper also gave sizing at the higher temperature but the degree of sizing was lower than that obtained in the presence of Tris-HC[1].

Example 2

The effect of incubation time on the levels of sizing imparted using a *Humicola insolens* cellulase preparation (Celluzyme®, Novo Nordisk A/S, Bagsvaerd, Denmark) was investigated.

Tris-HCl (50 mM, pH 7.5, 10 ml) was added to 0.2g pulp in distilled water and the *Humicola insolens* cellulase preparation (875 µl) was added and the mixtures incubated for either 15 min or 90 min at room temperature. At the end of the incubation period, the pulp samples were vortex mixed and diluted to 100 ml. Test papers were prepared in the standard manner and dried by a single pass through a drum drier 100°C/250 s.

The degree of sizing achieved by the different methods was assessed using the IDT method.

Table 2:

Enzyme	wt dry test paper (g)	IDT sizing	Incubation period (min)
Celluzyme® (875 µl)	0.207	+++	15
Celluzyme® (875 µl)	0.157	+++	90
Control	0.218	0	90

Increasing the incubation time from 15 min to 90 min did not significantly increase the level of sizing.

Example 3

To establish a quantitative relationship between sizing achieved in test papers and the quantity of *Trichoderma reesei* or *Humicola insolens* cellulase preparations added, a further set of experiments were undertaken.

Standardized paper making conditions were employed as follows: to a sample of pulp in distilled water (equivalent to 0.2 g dry paper) 10.0 ml buffer was added (50 mM Tris HCl, pH 7.5). Various amounts of cellulase (*Trichoderma reesei* or *Humicola insolens* ; Table 3) was added and the mixture vortex mixed. The pulp was incubated with gentle shaking at room temperature for 15 min, after which time the mixture was diluted to 150 ml with distilled water and the test paper sheets produced. Each test sheet was removed from the mesh and pressed between a folded sheet of dry 3 MM blotting paper using a hand-held roller. The sheet (still folded in the blotting paper) was passed once through a drum drier at 100-104°C with a 250 s contact time. The results are given in Table 3.

Table 3: Sizing achieved using either Trichoderma reesei (Celluclast®, Novo Nordisk A/S, Bagsvaerd, Denmark) or Humicola insolens (Celluzyme®, Novo Nordisk A/S, Bagsvaerd, Denmark) cellulase preparation

Celluzyme® (µl) as received	Celluzyme® (Binding protein added (based on weight of fibre) % w^{-1}	IDT sizing rating	Celluclast® (µl) as received	Celluclast® (Binding protein added (based on weight of fibre) % w^{-1}	IDT sizing rating
0	0	0	0	0	0
50	0.5	+	25	1.6	+
70	0.7	+	50	3.1	+
100	1.0	+	70	4.4	+
150	1.5	++	100*	6.3	+
200	2.0	+++	150	9.4	+

250	2.5	+++	200	12.5	+
300	3.0	+++	300	18.8	++
625*	6.2	+++	400	25.1	++
875	8.7	+++	600	37.6	++

*Represent equivalent addition levels.

The results show that whilst both cellulases impart sizing, the *H. insolens* cellulase preparation imparted greater levels of sizing than the *T. reesei* cellulase under the conditions used.

Example 4 The effects of buffer omission and high (160°C) temperature drying on levels of sizing achieved with either a *Trichoderma reesei* cellulase preparation (Celluclast®, Novo Nordisk A/S, Bagsvaerd, Denmark) or a *Humicola insolens* cellulase preparation (Celluzyme®, Novo Nordisk A/S, Bagsvaerd, Denmark) were investigated.

Paper sheets were prepared as described previously, with the various conditions as described in Table 4. Sizing was measured the following day by the standard IDT method.

The results show that Celluclast® benefits from higher drying temperatures (160°C) than Celluzyme® to confer good sizing.

Table 4: Effect of temperature on the sizing achieved by either Trichoderma reesei cellulase (Celluclast®, Novo Nordisk A/S, Bagsvaerd, Denmark) or Humicola insolens cellulase (Celluzyme®, Novo Nordisk A/S, Bagsvaerd, Denmark)

Enzyme (150 µl)	Binding protein added (based on weight of fibre) % ww^{-1}	Incubation buffer	Drying regime	IDT Sizing rating
Celluclast®	9.4	dH_2O	160°C/30 s + 108°C/250 s	++++
Celluclast®	9.4	Tris-HCl	160°C/30 s + 108°C/250 s	++

Celluclast®	9.4	dH_2O	108°C/250 s	+++
Celluclast®	9.4	Tris-HCl	108°C/250 s	+
No enzyme	0	Tris-HCl	160°C/30 s + 108°C/250 s	0
No enzyme	0	dH_2O	160°C/30 s + 108°C/250 s	0
Celluzyme®	1.5	dH_2O	160°C/30 s + 108°C/250 s	++
Celluzyme®	1.5	Tris-HCl	160°C/30 s + 108°C/250 s	++
Celluzyme®	1.5	dH_2O	108°C/250 s	++++
Celluzyme®	1.5	Tris-HCl	108°C/250 s	+

Example 5 The Hercules Sizing Test (HST) was used to determine the degree of sizing imparted by either *Trichoderma reesei* cellulase (Celluclast®, Novo Nordisk A/S, Bagsvaerd, Denmark) or *Humicola insolens* cellulase (Celluzyme®, Novo Nordisk A/S, Bagsvaerd, Denmark) with and without the addition of Tris. To prepare the sheets, 2 % (wv^{-1}) pulp stock was incubated with 5 % (ww^{-1}) cellulase protein (based on dry weight of fibre) for 5 min at 25°C before forming the paper sheets. The sheets were dried on a drum dryer at 105°C for 40, 80, 160 or 240s and were subjected to one of the following post manufacture heat treatments: naturally aged for 24 h; 80°C for 10 min and 105°C for 10 min.

The results show that *Humicola insolens* and *Trichoderma reesei* cellulases confer moderate sizing as measured by the HST after drum drying at 105°C.

Table 5 HST determination of paper sheets sized with either Trichoderma reesei cellulase (Celluclast®, Novo Nordisk A/S, Bagsvaerd, Denmark) or Humicola insolens cellulase (Celluzyme®, Novo Nordisk A/S, Bagsvaerd, Denmark).

		HST (s)					
		dH_2O			25 mmoles Tris		
Cellulase	Drum Drier Contact Time (s)	N/A	80°C	105°C	N/A	80°C	105°C
Celluclast®	40	10	13	18	10	11	15
	80	13	15	22	10	14	16
	160	19	18	23	16	16	20
	240	25	23	50	19	17	22
Celluzyme®	40	121	118	127	131	121	93
	80	137	141	149	134	128	104
	160	134	135	138	155	103	126
	240	111	106	117	112	98	113

Example 6 A Cellulase preparation from *Trichoderma viride* (BDH Ltd.) was tested as a biosizing agent. Samples were added to aliquots of pulp stock (0.2 g dry weight fibre in 15 ml distilled water). The cellulase addition level was adjusted such that an equivalent cellulose binding protein concentrations (corresponding to 8.7% ww^{-1} based on fibre weight) were added to enable direct comparison with *Humicola insolens* cellulase (Celluzyme®, Novo Nordisk A/S, Bagsvaerd, Denmark).

The pulp and cellulase samples were incubated at room temperature for 15 min prior to preparation of the hand sheets. The sheets were dried by a single pass through a drum drier at 105°C and left at room temperature overnight before testing for sizing using the IDT. The results are shown in Table 6.

The results indicate that when using an equivalent protein concentrations and compared with *Humicola insolens* cellulase, the *Trichoderma viride* cellulase imparted a moderate sizing effect.

Table 6 Sizing achieved by different cellulases as determined by the CHL-IDT assay.

Cellulase Source	Supplier	IDT sizing rating
Trichoderma viride	BDH Ltd.	+
Humicola insolens	Novo Nordisk A/S	+++
Control		0

Example 7 To test the application of Clostridium *thermocellum* cellulosomes as a sizing agent *Cl. thermocellum* (NCIMB 10682) was grown on 1.0 % (wv^{-1}) pulp in growth medium, comprising: 1000ml Basal Medium ((gl-1) yeast extract, 10; KH_2PO_4, 1.5; $K_2HPO_4.3H_2O$, 2.9; $(NH_4)_2SO_4$, 1.3; and $FeSO_4.7H_2O$, (1.0% wv^{-1}) 1.0ml^{-1}) with a cellulose source (pulp @1% wv^{-1}) which is then autoclaved to sterilize. 25 ml Salts Solution ($MgCl_2$, 2%wv^{-1}; $CaCl_2$ 0.2% wv^{-1}, autoclaved to sterilize). 15 ml Cysteine Solution (50 gl^{-1}, filter sterilized). The growth medium (1l in a Duran bottle) was purged with nitrogen for 5 min and heated to 60°C prior to inoculation. The culture was then incubated for 240h. The culture was harvested and the cells and pulp debris separated from the culture fluid by centrifugation. Sodium azide (0.02 % (wv^{-1})) was added to both the culture fluid and to the pulp debris to prevent further microbial growth.

The culture fluid was tested as a sizing agent by making paper using the *Cl. thermocellum* cultures. Water-leaf pulp (10% (wv^{-1}); 2.16 g) was weighed into five 250 ml flasks. *Cl. thermocellum* culture fluid (200; 100; 50; 25; and 0 ml) was then added to each flask and the volume adjusted to 200 ml with distilled water. The mixture was then stirred at room temperature for 15 min and a paper sheet was made from the contents of each flask using the standard paper making method. The paper was dried at 80°C for 250 s using the drum drier. Sizing was measured the following day using the standard IDT method.

Control sheets of paper were also prepared and tested as

above wherein non-inoculated *Cl. thermocellum* growth medium was added to the water-leaf pulp instead of the *Cl. thermocellum* culture fluid.

It was also decided to test for sizing using the pelleted pulp debris and cells. The pulp debris and cells (2.16 g; percentage pulp now unknown) were weighed into two 250 ml flasks. To one flask 200 ml of *Cl. thermocellum* growth medium was added to resuspend the pulp debris and to the other flask 200 ml of distilled water was added. Both flasks were stirred at room temperature for 15 min and a paper sheet made from the contents of both flasks using the standard paper making method. The paper was dried at 90°C for 250 s using the drum drier. Sizing was measured the following day using the IDT method.

The *Cl. thermocellum* culture fluid did not impart sizing to the paper it is believed because of the very low levels of cellulosomes free in the culture fluid. Paper sheets made from the pulp debris showed sizing (Table 4) and a significant lowering in the degree of sizing was noted when distilled water was used compared to the *Cl. thermocellum* growth medium. It is believed that the use of distilled water causes a lowering of the salt/ionic strength of the distilled water as compared to use of the growth medium, resulting in elution of cellulosomes from the pulp surface thereby reducing the degree of sizing. The results confirm that the presence of the *Cl. thermocellum* cellulosome preparation imparts sizing to the paper sheet.

Table 7: Sizing test: pulp hydrolysed by Cl. thermocellum

	Diluent (ml)	Ink-drop test (s)
Pulp debris + cells (2.16 g)	*Cl. thermocellum* growth medium (200 ml)	140/175*
Pulp debris + cells (2.16 g)	dH_2O (200 ml)	37/35*

* Duplicate tests.

Example 8

A further series of experiments were performed to determine the effect of cellulase on the sizing of paper. In the experiments, the following materials and general protocols were employed:-

Cellulase

An aqueous *Trichoderma reesei* cellulase preparation was employed ("Celluclast 1.5L" supplied by Novo Nordisk Bioindustry S.A. 92017 Nanterre Cedex. France).

In addition Cellulase derived from *Penicillium funiculosum* available as a tan powder from Sigma Aldrich Co. Ltd. Poole, Dorset, U.K. was used.

Stock Preparation

Except where otherwise indicated, the furnish used was a blend of ECF bleached hardwood and softwood pulps (ratio of 70:30 HW/SW). The stock was prepared with $^1/_3$ PBS and no fillers were added. The procedure was as follows:

280g of bleached hardwood pulp and 120g of bleached softwood pulp were added to 18 litres of $^1/_3$ PBS. The fibres were dispersed by vigorous agitation. This stock was then transferred to the Hollander and beaten until a freeness value of 25oSR was attained (time taken was usually 30 to 35 minutes). The stock was then adjusted to a final consistency of 2% with further $^1/_3$ PBS as necessary.

Addition/Incubation of Additives

The cellulase solution was added to the thick (2% consistency) stock. Two litres of the thick stock (containing 40g of fibre) was contained in a metal jug and stirred at the lowest possible speed to achieve a slow movement of the stock. Vigorous agitation should be avoided otherwise denaturing of the enzyme may occur during the incubation period. The stock was at ambient

temperatures (20-25°C).

The incubation time was fifteen minutes. During this incubation period the movement of the stock may appear to become easier/faster. If this is apparent then reduce the stirrer speed as much as possible.

After the fifteen minute incubation period had elapsed the thick stock was then added to the proportioner.

Proportioner

The thick stock in the proportioner was then diluted to a consistency of 0.25% using DEMI water only. Normal agitation speeds in the proportioner were employed to mix the stock.

Handsheet Formation

The white water box was filled with DEMI water for handsheet formation. With the handsheet forming wire in place in the mould assembly, one litre of stock from the proportioner was added to the Deckle Box, together with water from the white water box. The contents of the Deckle Box were agitated with the perforated agitator (moved up and down five times). After the fifth stroke the agitator was rested on the surface of the water to help dampen the motion of the water in the Deckle Box. The water was then pumped back to the white water box and the initial wet mat was formed.

Depending on how vigorous the agitation has been some foaming may occur in the Deckle Box. This foam may still persist after the initial wet mat is formed and can be quite substantial. Some of this foam can be dispersed if the pump is kept on for a few seconds after the water has been removed so that air can be drawn through the mat.

Handsheet Pressing and Drying

The wet mat and handsheet wire were removed from the mould to the press. The moisture content of the pressed sheet should be 70%. The pressed sheet was then dried on an electrically heated drum dryer. The surface temperature of the dryer was 105°C and the speed of the dryer was such that the pressed sheet was in contact with the hot surface for 35 seconds. The final moisture content of the sheet should be between 4 and 7% (typically 5%).

If the moisture content of the sheet after pressing is less than 70%, then the sheet may stick to the surface of the drum dryer when the above conditions are employed. This may occur because of nonuniform press pressures being applied across the width of the sheet. Steps should be taken to avoid this.

When the surface temperature of the drum dryer is less than 105°C but is 70°C or higher, longer contact times are required in order for the handsheet to have a final moisture content of 5%.

If the surface temperature of the drum dryer is below 70°C, it is necessary to extend the contact time further or increase the initial pressing on the wet mat to remove more water or to do both. It is possible to reduce the moisture content of the pressed sheet to less than 60%.

Testing

Conditioning and testing of the paper is done according to procedures laid out in the "Tappi Test Methods" published by TAPPI, Technology Park Atlanta, PO Box 105113, Atlanta GA 30348, USA, ISBN 0 - 89852 - 200 - 5 (vol 1 and 2). The HST (Hercules Sizing Test) is defined as size test for paper by ink resistance T 530 pm - 83; and the Cobb test is defined by T 441 om - 84.

A series of experiments were performed in which the cellulase concentration, aging time and temperature were each varied. The results are presented in the following tables in which:-

"naturally aged" refers to storage for the specified time at 23°C ± 1°C in relative humidity 50.0 ± 2% as specified in T402om-83;

"oven cured" refers to treatment at 80°C for 30 minutes.

Sizing Performance of Handsheets made with Cellulase and dried under standard conditions

"HST (seconds) of handsheets made with *Penicillium funiculosum*"

Protein added	Ageing Condition		
	24h naturally aged	2 weeks naturally aged	oven cured
blank	1	1	1
10%	1	7	6
15%	3	11	18

"HST (seconds) of handsheets made with *Trichoderma reesei*"

Protein added	Ageing Condition		
	24h naturally aged	2 weeks naturally aged	oven cured
blank	1	1	1
5%	13	27	28
10%	34	53	61

"Cobb (gsm) of handsheets made with *Trichoderma reesei*"

Protein added	Ageing Condition		
	24h naturally aged	2 weeks naturally aged	oven cured
blank	Fully saturated	Fully saturated	Fully saturated
5%	78.6	67.2	74.2
10%	68.5	51.2	50.7

Example 9

In a further series of experiments conducted under the protocols described in Example 8 above, the degree to which the added protein [Celluclast (*Trichoderma reesei*, Novo Nordisk) and Celluzyme (*Humicola insolens*, Novo Nordisk)] is retained by the paper was investigated.

In separate experiments Celluclast and Celluzyme were added to paper pulp and test sheets prepared described above (24 h naturally aged). The amount of protein retained in the paper (as opposed to that remaining in the pulp supernatant when forming the paper web) was estimated on the basis of the nitrogen content of the paper, assuming that the nitrogen content of both proteins is 16% w/w. The nitrogen content was measured by Antec micropyrolysis.

The results are presented in the following table:

Additive	Addition level w/w db fibre	% Nitrogen in dry paper w/w	% Protein retained in paper w/w db fibre	HST (seconds)	Cobb (g_2 water/m^2
Blank	-	0.007	-	1	fully saturated
Celluclast	20	0.356	2.225	273	15.5
Celluzyme	5	0.374	2.3375	263	28.6

The results show that addition levels of Celluzyme one quarter those of Celluclast gave rise to similar protein retention levels. At similar protein retention levels, both celluloses gave similar sizing effects.

Example 10

In the following example, the binding amylase enzymes to starch is demonstrated. Two amylase enzymes were characterized using HPLC: an α-amylase (Type X-A crude preparation) from *Aspergillus oryzae* and amyloglucosidase from *A. niger* (available from Sigma Aldrich Co. Ltd., Poole, Dorset, United Kingdom). The main catalytic peaks of each preparation were determined using a starch glucose-release

assay. The binding efficiencies of each protein were determined against a range of starches with BSA controls included in the assessment.

A solution of 32 mg ml^{-1} (dry weight) of α-amylase was made up in 0.1 M PBS (pH7.0). 100μl of this was loaded onto an HPLC using a Bio-Sil SEC gel permeation column running 0.1 M phosphate buffer at 1 ml min^{-1}. Fractions (1 ml) were collected and tested for reducing sugars released from a starch suspension using the standard microtitre assay (for glucose).

The following qualitative assay was used to detect glucose and cellobiose in test samples. The assay was carried out in a micro titre dish at room temperature.

Reagent Components:

10 μl phenol reagent (0.128M phenol in 0.1M phosphate buffer pH7.0)
10 μl amino pyrine reagent (19.7mM 4-amino phenazone in 0.1M phosphate buffer pH7.0)
10 μl peroxidase in 0.1M phosphate buffer pH 7.0 (to give 800Eu/ml)
10 μl glucose oxidase in 0.1M phosphate buffer pH 7.0 (to give 250Eu/ml)
60 μl 0.1M phosphate buffer pH7.0

These reagent components were mixed and added to the wells of a microtitre dish. Test samples 100 μl were added followed by an excess of substrate (starch). The appearance of a red colour was indicative of the presence of amylase.

The same methods were also used to produce an HPLC profile for the amyloglucosidase. The amyloglucosidase was a liquid preparation containing approximately 262 mg ml^{-1} protein as measured by the Coomassie Blue technique. 100 μl of a 0.007 dilution in 0.1 M PBS (pH 7.0) was loaded onto the HPLC and

monitored at 230 nm 0.1 AUS. 1 ml fractions were collected and tested for reducing sugars released from starch suspensions as above.

The ability of α-amylase and amyloglucosidase to bind to normal starch in suspension was assessed. Starch (0.2 g; Roquette) was added to 9 ml 0.1 M PBS (pH 7.0) and 1 ml α-amylase solution (9.5 mg ml^{-1} by Coomassie Blue assay) was added. This was incubated on a shaker for 20 min.

The sample was centrifuged at 13,000 rpm for 5 min and 100 µl samples loaded onto the HPLC column. The peak profile of the 20 min bound α-amylase was compared with a T = 0 sample. From this data the percentage binding of the enzyme was calculated. The binding of amyloglucosidase was also tested against cationic starch. BSA was also used in the same way as a control. The final concentration of the BSA used was 0.2% (wv^{-1}) in 0.1 M PBS.

The results of the binding experiments are shown in the following Table.

Starch binding profiles

Enzyme	Substrate	% Bound
α-amylase	starch	32
amyloglucosidase	starch	27
amyloglucosidase	cationic starch	45
BSA	starch	7
BSA	cationic starch	6

These results indicate that both α-amylases and amyloglucosidases specifically bind to both starch and cationic starch.

CLAIMS

1. A method of sizing paper comprising the steps of a) contacting said paper or a constituent of said paper with a protein capable of binding to said paper or said constituent of paper; and b) denaturing or heating said protein bound to said paper.

2. A method of sizing paper comprising the steps of a) contacting said paper or a constituent of said paper with a protein capable of binding to said paper or said constituent of paper; and b) heating said paper.

3. A method according to claim 2 wherein the paper is heated to a temperature of 70°C to 170°C.

4. A method according to claim 3 wherein the paper is heated to a temperature of 80°C to 110°C.

5. A method according to any preceding claim wherein the protein is capable of binding a polysaccharide.

6. A method according to any preceding claim wherein the protein is capable of binding cellulose.

7. A method according to any preceding claim wherein the protein is a cellulase or fragment thereof.

8. A method according to claim 7 wherein the protein is a cellulase selected from the group comprising *Cellumonas fimi*, *Trichoderma viride*, *Trichoderma reesei*, *Aspergillus*

niger, *Fusarum Oxysporum*, *Penicillium funiculosum* and *Humicola insolens*.

9. A method according to claim 7 wherein the protein is an exo cellulase or fragment thereof.

10. A method according to claim 9 wherein the protein is cellulase derived from *Humicola isolens*.

11. A method according to claim 7 wherein the protein is an endo cellulase or fragment thereof.

12. A method according to claim 11 wherein the protein is cellulase derived from *Trichoderma reesei*.

13. A method according to any one of claims 1 to 5 wherein the protein is capable of binding starch.

14. A method according to claim 13 wherein the protein is an amylase or fragment thereof.

15. A method according to claim 1 wherein the protein is denatured by means of heat.

16. A method according to claim 1 wherein the protein is denatured by means of treatment with a chemical denaturant.

17. A method of manufacturing sized paper comprising the steps of a) preparing a paper pulp, b) adding a protein

capable of binding to a constituent of said pulp, c) forming paper from said pulp, and d) heating the paper.

18. A method of manufacturing sized paper comprising the steps of a) applying to paper a protein capable of binding said paper and b) heating said paper.

19. Paper sized according to a method of any one of claims 1 to 18.

20. Use of a protein capable of binding to paper or a constituent of paper for the purpose of sizing paper.

INTERNATIONAL SEARCH REPORT

Intern al Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 D21H17/22 D21H21/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 D21H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 980 023 A (SALKINOJA-SALONEN MIRJA) 25 December 1990 see the whole document ---	1
A	US 3 222 245 A (POSCHMANN FRANZ) 7 December 1965 see the whole document -----	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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